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THE INCREASE IN MITOCHONDRIAL DNA COPY NUMBER IN THE TISSUES OF Γ-IRRADIATED MICE

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Abstract: Changes in the number of mitochondrial DNA (mtDNA) copies in the brain and spleen tissues of gamma-irradiated (3 Gy) mice were studied by comparative analysis of the long-extension PCR products of mtDNA (15.9 kb) and a fragment of the cluster nuclear β-globin gene (8.7 kb) amplified simultaneously in one and the same test-tube within total DNA. The analysis showed that, compared to the nuclear β-globin gene, an increase in mtDNA copy number (polyploidization) took place in the brain and spleen cells of mice exposed to gamma-radiation. This data led to the suggestion that the major mechanism for maintenance of the mitochondrial genome, which is constantly damaged by endogenous ROS and easily affected by ionizing radiation or other exogenous factors, is the induction of synthesis of new mtDNA copies on intact or little affected mtDNA templates because the repair systems in the mitochondria function at a low level of efficiency.

Key Words: Amplification of Mitochondrial DNA, γ-Irradiated Mice, LX-PCR Assay

INTRODUCTION

The results of a number of studies indicate that mtDNA is damaged more frequently than nuclear DNA (nDNA) by the reactive oxygen species (ROS) generated by the mitochondria themselves. Chemical agents and ionizing radiation (IR) also produce more lesions to mtDNA than to a comparable nDNA fragment in the cell [1-4]. Moreover, the various DNA repair systems either do

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Abbreviations used: nDNA – nuclear DNA; mtDNA – mitochondrial DNA; ROS – reactive oxygen species; IR – ionizing radiation; LX-PCR – long-extension PCR.

not function at all or function at a low level of efficiency in the mitochondria [2, 3, 5-7]. However, it has been shown that mtDNA biosynthesis in the cells and tissues of lethally irradiated mammals is not suppressed, whereas the replication of nDNA under the same conditions is inhibited [8-12].

Apparently, the increased number of mtDNA lesions and the low efficiency of their repair, and the absence of a replication block on damaged DNA in the mitochondria considerably contribute to the formation of mutations in mtDNA, at a frequency 10 to 25-plus times that seen in nDNA [3, 13-15]. The formation of deletions has also been observed in the mtDNA of cells exposed to IR [16-18]. Increased levels of ROS in the mitochondria and the action of exogenous damaging agents induce structural alterations in the mitochondrial genome or its degradation, and hence disturbances in ATP synthesis [1, 13-15]. Therefore, it is important to establish what mechanisms perform maintenance of the mitochondrial genome in cells exposed to damaging agents, especially considering the low-efficiency of the repair systems functioning in the mitochondria. The results of some investigations on cell cultures indicated that, besides damage to and structural rearrangements of the mtDNA, in cells under increased oxidative stress and affected by other damaging agents, biogenesis of mitochondria and/or mtDNA polyploidization occur [19-24].

The action of IR may result in a significant ATP deficiency in the cells not only because of damage to the respiratory chain control genes but also due to the activation of poly(ADP-ribosyl)ation of the nuclear proteins [25]. It is probably the occurrence of ATP deficiency that causes mtDNA polyploidization in irradiated cells. This assumption is consistent with the data on increased mtDNA synthesis (registered by radioautography) and the activation of mtDNA gene expression after the exposure of cells to IR [26, 27].

Thus, the investigation of mtDNA damage and biogenesis induction in the tissues of whole-body irradiated mammals is of considerable interest. In this investigation, we studied changes in mtDNA in the brain and spleen tissues of gamma-irradiated mice by comparative assays of the products of long-extension PCR (LX-PCR) of mtDNA and the β-globin nuclear gene amplified in one and the same test-tube within total DNA. The assays showed an active increase in the number of mtDNA copies relative to those of the nuclear β-globin gene in the brain and spleen tissues of gamma-irradiated mice in the post-irradiation period.

MATERIALS AND METHODS

Animals and preparation of subcellular fractions

Two-month old male BALB/c mice from the nursery of experimental animals at the Pushchino Branch of the Institute of the Bioorganic Chemistry, RAS, were used. The mice were kept under standard nursery conditions. Each experimental group contained 5-6 mice. Irradiation was carried out on a gamma-installation (^{137}Cs) at a dose rate of 1.5 Gy/min. The mice were killed by cervical dislocation. Brain (hemispheres) and spleen tissues were taken for assay. All the

manipulations were performed at 2-3ºC. Tissue pieces were placed in buffer A (250 mM sucrose, 50 mM Tris-HCl, 2 mM dithioerythritol, 0.5 mM PMSF, 50 mM KCl, 5 mM $MgCl_2$, pH 7.5) in the ratio 1:5 (tissue weight:buffer volume) and homogenized in a glass homogenizer with a teflon pestle. The cell nuclei and mitochondria fractions were isolated from the homogenates by differential centrifugation. The nuclei were purified by centrifugation in buffer A containing 2 M sucrose. The mitochondria were additionally purified by centrifugation in a percoll gradient as indicated in [28]. The nuclei and mitochondria were resuspended in buffer B (250 mM sucrose, 10 mM Tris-HCl, 0.5 mM PMSF, 50 mM KCl and 2 mM EDTA, pH 7.5) and DNA was isolated.

DNA purification and performance of LX-PCR

The total DNA from tissue homogenates and DNA from separate suspensions of nuclei and mitochondria were isolated as described in [29]. DNA samples were settled by ethanol and kept at -20ºC for one month. For analysis, the DNA was dissolved in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the concentration was measured spectrophotometrically.

The LX-PCR of the mtDNA (15.9 kb) and the β-globin gene fragment (8.7 kb) was carried out in one and the same test-tube. The primers for the PCR of the mtDNA were MSFIFor (5'-TTT ATA GGC TAC GTC CTT CCA TGA GG-3') and MSFIRev (5'-GGC AGG TAG GTC AAT GAA TGA GTG G-3') [30]. To amplify the β-globin gene fragment, primers 21582 (5'-TTG AGA CTG TGA TTG GCA ATG CCT-3') and 30345 (5'-CCT TTA ATG CCC ATC CCG GAC T-3') [31] were used. The LX-PCR of the mtDNA and of a β-globin fragment was performed with the use a mixture of *Taq* and *Pfu* DNA polymerases (Fermentas UAB, Lithuania). The reaction mixture for LX-PCR (total volume 25 to 50 μl) contained different amounts of DNA template, 200 nM of primers of each pair, 300 μM of each dNTP, 2.5 mM $MgCl_2$, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.01% twin 20, pH 8.8, and 1.0 unit of a total mixture of *Taq* and *Pfu* DNA polymerases, which was introduced by the method of "hot start" after the primary denaturation at 94ºC for 4 minutes. The LX-PCR amplification was carried out in 25 to 29 cycles as follows: denaturation for 10 sec at 94ºC, and annealing and elongation for 12 min at 68ºC. After completion of the above cycles, the reaction mixtures were additionally incubated for 10 min at 72ºC. All the amplification procedures were done on a Perkin-Elmer 9700 thermocycler.

The PCR products were visualized on a UV-transilluminator after electrophoresis in a 0.8% agarose gel with ethidium bromide. The PCR products on electrophoregrams were scanned and digitized with an ID-PRINT optical system ("IAP", S.-Petersburg). In a comparative assay, the ratios of PCR amplification product intensities of the mtDNA (A) to those of the nuclear β-globin gene fragment (B) were determined [32]. The results were expressed as the means ± S.D.

RESULTS

It was previously shown in a number of studies that lesions in DNA templates can block thermostable DNA polymerases in PCR, which results in a decreased yield of amplification products from the DNA samples [31, 33-36]. This enabled the use of the PCR method in assessing damage to individual genes and made it possible to study gene-specific repair. We were able to demonstrate a decrease in the amount of PCR products of gamma-irradiated DNA templates, caused by radiation-induced breaks, AP sites and modified bases [35, 36]. The results of the above-cited and other studies demonstrated that PCR could be used to assess changes in the copy number of intact DNA templates of equal sequence. For comparative assays of mtDNA and nuclear gene copy numbers, special conditions were chosen to conduct LX-PCRs of mtDNA of 15.9 kb [30] and an almost half as small β-globin nuclear gene fragment (8.7 kb) [31] within total DNA in one and the same test-tube. Fig. 1 shows the amplification products of separate samples of mtDNA and nDNA isolated from cell organelles and the total DNA from mouse spleen tissue. It is seen that the conditions chosen for LX-PCR with total DNA enabled us to obtain the same products as in PCRs with individual DNA templates isolated from mitochondria and nuclei. To compare the PCR products of mtDNA and β-globin gene fragment, total DNA samples were introduced to the reaction in amounts that provided a linear dependence of LX-PCR product yield on DNA template concentration in the reaction mixture.

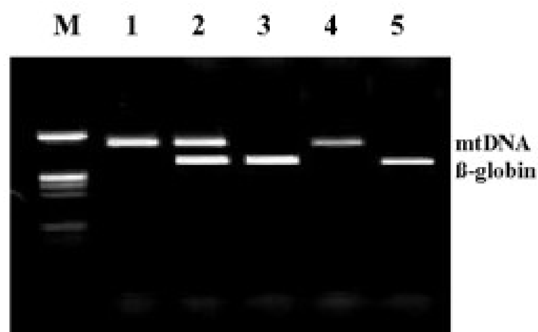

Fig. 1. The products of combined and separate LX-PCR amplification of mtDNA (15.9 kb) and a β-globin gene fragment (8.7 kb). DNA samples from mouse spleen tissue and appropriate primers were used in LX-PCR: lane 1 – total DNA, mtDNA primers; lane 2 – total DNA, mtDNA- and β-globin gene primers; lane 3 – total DNA, β-globin gene primers; lane 4 – mtDNA, mtDNA primers; lane 5 – nuclear DNA, β-globin gene primers. Here and in the other Figs, lane M – molecular weight marker: Lambda DNA/*EcoR* I + *Hind* III (Fermentas UAB, Lithuania).

As shown in Fig. 2, with 0.5-5.0 ng of total DNA introduced to PCR and with 27 amplification cycles, the PCR products of mtDNA and the β-globin gene and their ratios could be characterized quantitatively (in relative units). At least in

the range of 0.5-10 ng DNA per 50 μl of total reaction mixture and with 27 amplification cycles, we obtained a linear dependence of PCR product yield on the DNA template amount. In the reaction conditions chosen, the level of PCR products of gamma-irradiated DNA samples is solely dependent on the number of copies of intact DNA-templates.

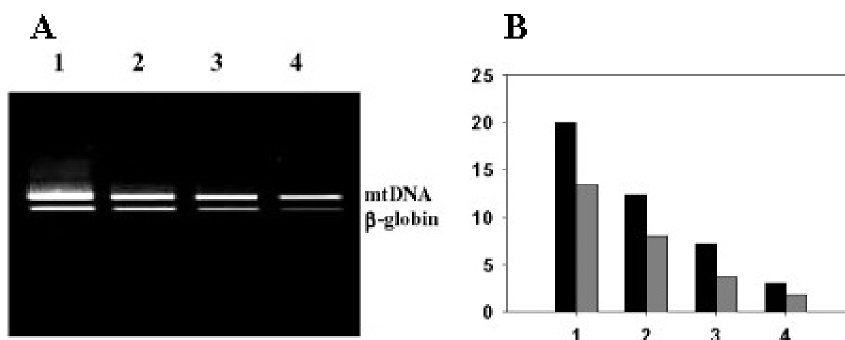

Fig. 2. The LX-PCR amplification products of mtDNA and a β-globin gene fragment within total DNA of mouse brain tissue: agarose gel stained with ethidium bromide (A); the results of scanning the same gel and digitizing each PCR product in the lanes (B). LX-PCR was carried out in 27 cycles. The ordinate is the PCR product intensity. The abscissa is the amount of total DNA (ng) introduced to the reaction: lane 1 – 5.0; lane 2 – 2.5; lane 3 – 1.0; lane 4 – 0.5.

With equal DNA amounts introduced to the reaction mixture, a decrease or an increase in the amount of PCR product are indicative of changes in the number of intact copies of amplified sequences. On the other hand, if the nuclear β-globin gene fragment is regarded as an internal standard (for comparison with mtDNA) in its amplification simultaneously with mtDNA in LX-PCR, the increase in the amount of mtDNA amplification product relative to the amount of β-globin gene fragment PCR product indicates an increase in mtDNA copy number. The data presented in Fig. 3 indicates that the levels of mtDNA and β-globin gene fragment PCR products substantially differ between the *in vitro* irradiated and intact total DNA preparations. The differences are probably due to both different copy numbers of mtDNA and β-globin gene fragments within the total DNA, and different amounts of radiation-induced lesions in these DNA fragments. Probably, less radiation-induced lesions are formed in a β-globin gene fragment of 8.7 kb than in an mtDNA fragment of 15.9 kb.

Thus, the results presented in Figs 1-3 suggest that the LX-PCR method can be used to assess the induction of mtDNA polyploidization in the tissues of irradiated animals.

When LX-PCR was carried out with total DNA purified from the brain and spleen tissues of mice just after gamma-irradiation, the decrease in the ratio of mtDNA to β-globin gene amplification products was not very marked (Fig. 4).

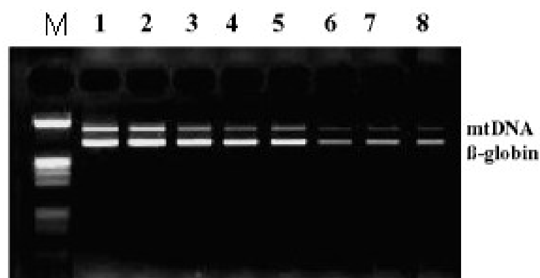

Fig. 3. The LX-PCR products of mtDNA and a β-globin gene fragment. Total DNA samples from mouse spleen tissue were used. Lanes 1, 2 – non-irradiated; lanes 3-5 – irradiated *in vitro,* 5 Gy; lanes 6-8 – irradiated *in vitro,* 10 Gy.

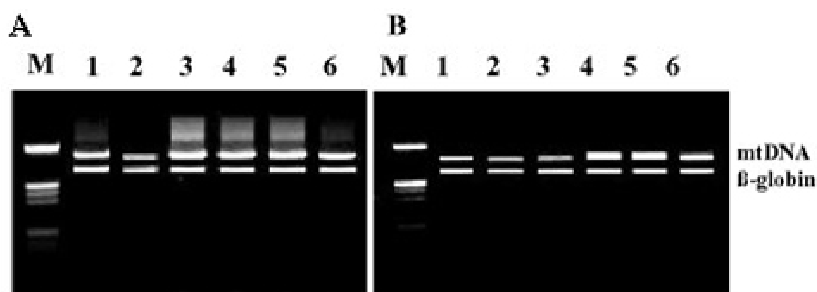

Fig. 4. The LX-PCR amplification products of mtDNA and a β-globin gene fragment within total DNA from brain (A) and spleen (B) tissue from gamma-irradiated (3Gy) mice. Lane 1 – non-irradiated animals; lane 2 – just after irradiation; lanes 3-6 – 24, 48, 72, and 96 hours after irradiation, respectively.

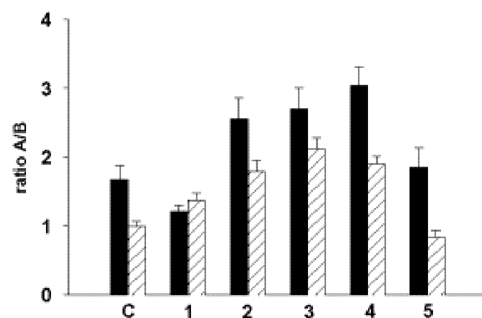

Fig. 5. The change in mtDNA copy number compared with that for a nuclear gene fragment in the tissues of mice exposed to gamma-radiation at a dose of 3 Gy. Dark columns are for the brain, hatched columns are for the spleen. The ordinate shows the ratio of the LX-PCR product intensity of the mtDNA (A) to that of the β-globin gene fragment (B). The abscissa shows animal killing times after irradiation: C – control; 1 – just after irradiation; 2-5 – respectively 24, 48, 72 and 120 hours after irradiation.

This can be explained by the small amount of primary lesions arising in the mtDNA fragment (about 16 kb) after exposure of the mice to 3 Gy. Nevertheless, in subsequent assays, 24-96 h after irradiation, we observed a significant increase in the amount of mtDNA amplification product compared with the level of nuclear gene product (Fig. 4).

This indicates an increase in the copy number of mtDNA against that of the β-globin gene within the total DNA. It should be noted that by this time after the irradiation of the mice (24-96 h), the majority of nDNA lesions had been repaired. Therefore, the β-globin gene fragment can be regarded as a stable standard in its simultaneous PCR amplification with mtDNA within the total DNA isolated from the tissues of the control and irradiated mice. The results of assays performed with total DNA purified from the tissues of the mouse groups (5 mice in each) are summarized and presented in Fig. 5. The data suggests that a significant increase in mtDNA copy number occurs in the post-mitotic (brain) and mitotically active (spleen) tissues of irradiated animals (Fig. 5). It can be concluded from these findings that in the brain and spleen tissue cells of mice exposed to non-lethal gamma-radiation, the mtDNA copy number increases compared with the nDNA copy number in the post-irradiation period, which may be due to a radiation-induced polyploidization of mtDNA in the tissues of these animals.

DISCUSSION

Studies on cultivated cells show that IR induces lesions in mtDNA at a higher frequency than in nDNA [4]. In mitochondria, the base excision repair system functions at a low efficiency, as reported by many investigators [2, 5-7]. The high level of mtDNA damage and the low repair efficiency probably substantially contribute to the higher level of mutations and deletions in the mitochondrial genome than in the nuclear genome [3, 13-18]. We can assume that the higher frequency of rearrangements in the mitochondrial genome of cells exposed to oxidative stress or ionizing radiation is determined not only by the high level of mtDNA lesions and the low efficiency of their repair, but also by the apparent absence in the mitochondria of control mechanisms for the inhibition of DNA replication until DNA repair has been completed. It is well known that mtDNA can replicate independently of the proliferative activity of cells and replication of nDNA. While the synthesis of nDNA in animal cells occurs during a limited period of the cell cycle, in the S-phase, the synthesis of mtDNA occurs in all the phases of the cell cycle [37]. When nDNA is damaged, its replication is blocked by the inducible system of the cell cycle checkpoint up to the completion of repair [38]. However, the replication of damaged mtDNA is evidently not blocked, although the frequency of lesions in it can be higher than in nDNA [8-12]. Obviously, the absence of mechanisms for blocking the replication of damaged DNA in the mitochondria largely promotes the formation of mtDNA mutations. At the same time, heavily damaged mtDNA copies are eliminated from these organelles to the cytosol [39].

Although lesions are formed in mtDNA of gamma-irradiated (3 Gy) mice, their amount is apparently not insufficient for the decrease in the amount of PCR product to be registered under the conditions chosen. The frequency of DNA lesions in "relaxed", actively transcribed portions of the nuclear chromatin is known to be substantially higher than in DNA fragments within an inactive, more condensed heterochromatin [40]. The vulnerability of mtDNA molecules to damaging agents possibly depends on their structural-functional organization and folding. As is known, each mitochondrion in a mammalian cell contains 2 to 10 mtDNA copies, which can form nucleoids and be bound to the proteins of the internal membrane of the organelle [41-43]. In mitochondria, numerous proteins have been found, including histone-like ones capable of forming complexes with mtDNA [44, 45]. Although these proteins do not form nucleosomal structures with mtDNA, they can cause a compact folding of the mitochondrial genome and protect it against ROS. Alternatively, it can be supposed that the transcribed mtDNA copies have less compact, relaxed folding patterns and hence are more available for the direct action of free radicals, whether of endogenous origin or induced by IR. Therefore, it is felt that transcribed and less compact mtDNA copies are most of all damaged in irradiated cells. In this study, we demonstrated an increase in the number of mtDNA copies within total DNA isolated from the brain and spleen tissue of irradiated mice. This increase in mtDNA copy number is most probably associated with the activation or amplification of intact or little damaged mtDNA molecules, which at the time of irradiation were within DNA-protein complexes and were thus protected from free radicals. The signal mechanisms for mtDNA amplification induction and the biogenesis of mitochondria in cells affected by damaging factors are as yet poorly understood [46-48]. The activation of these processes in the cells of humans and animals exposed to DNA-damaging agents or oxidative stress is considered a compensatory reaction of these cells to the energy deficiency caused by mtDNA damage or mutation induction [19-24]. The increase in mtDNA copy number found in the brain and spleen tissue of irradiated mice can be associated with an ATP deficiency arising in the cells of these tissues as a result of damage to a part of the mtDNA copies. Besides, it is well known that the reaction of poly(ADP-ribosyl)ation of nuclear proteins in IR-exposed cells is extraordinarily activated. Such protein modification needs NAD^+, the production of which requires substantial amounts of ATP [25]. The increase in mtDNA copy number and in biogenesis of mitochondria is probably necessary to compensate the energy deficiency occurring in the tissues of irradiated animals. It has been demonstrated that nitrogen oxide is an efficient inductor of biogenesis of mitochondria in a variety of cells and may influence the energy balance regulation in the organism [20, 21]. An increase in mtDNA copy number and mitochondrial mass has been observed after the exposure of cells to chemical oxidative agents and in connection with the mitochondrial dysfunction in tissues during the therapeutic treatment of virus pathologies [19, 22, 24]. The elevated level of mtDNA copies is revealed in the case of inheritable mitochondrial pathologies related to mtDNA mutations and deletions [23, 24, 49, 50].

Thus, it can be concluded that in mammals exposed to IR or other stress influences resulting in mtDNA damage, an increase in mtDNA copy number (polyploidization) takes place in the post-irradiation period. The activation of mtDNA amplification in the tissues of irradiated mammals is an essential process needed to compensate the energy deficiency caused by the radiation-induced damage to a part of the mtDNA copies. The data also led to the assumption that the major mechanism for maintenance of the mitochondrial genome, which is constantly damaged by endogenous ROS and affected by IR or other exogenous factors, is the induction of synthesis of new mtDNA copies on intact or little affected mtDNA templates because the repair systems in mitochondria are functioning at a low level of efficiency.

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